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Five new *Escovopsis* species from Argentina

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Abstract—*Escovopsis atlas*, *E. catenulata*, *E. longivesica*, *E. primorosea*, and *E. pseudoweberi* spp. nov. are described from fungal gardens of higher attine ants in Argentina. All these species possess clear diagnostic features: *E. atlas* characterized by subglobose vesicles; *E. catenulata* by the presence of claviform vesicles forming short chains; *E. longivesica* by longer vesicles than the rest of the species; *E. primorosea* by pink coloration of colony in mCYA medium; and *E. pseudoweberi* by the smallest conidia known in the genus.

Key words—*Acromyrmex*, ant nest parasite, *Atta*, *Hypocreales*, taxonomy.

Introduction

Escovopsis J.J. Muchovej & Della Lucia (*Hypocreales*) is a symbiotic genus of mycoparasites of attine ant nests (Currie & al. 2003). Muchovej & Della Lucia (1990) proposed *Escovopsis* as a substitute for the invalid genus "*Phialocladus*" Kreisel, which lacked a designated type (Kreisel 1972). The authors designated *Escovopsis weberi,* isolated from a fungal garden of *Atta* sp. in Minas Gerais, Brazil, as the type species of the genus. Seifert & al. (1995) described a second

species, *E. aspergilloides* Seifert & al., isolated from a nest of *Trachymyrmex* from Trinidad; they clarified the ontogeny in the genus and confirmed that the conidiogenous cells of *Escovopsis* species were phialidic, in agreement with Kreisel's (1972) original description of "*Phialocladus*".

Augustin & al. (2013) published three new species from nests of *Acromyrmex* in Minais Gerais, Brazil: *Escovopsis lentecrescens* H.C. Evans & J.O. Augustin, *E. microspora* H.C. Evans & J.O. Augustin, and *E. moelleri* H.C. Evans & J.O. Augustin; they also described *Escovopsioides* H.C. Evans & J.O. Augustin as a morphologically and molecularly distinct genus also associated with attine ant nests. Augustin & al. (2013) laid the groundwork for the current molecular taxonomy of *Escovopsis,* including new species and comparing them with previously described morphotypes from ecological and evolutionary research (Currie & al. 2003, Taerum & al. 2007, Gerardo & al. 2006).

Two additional *Escovopsis* species have been published: *E. kreiselii* L.A. Meirelles & al. from a nest of *Mycetophylax morschi* in Santa Catarina, Brazil; and *E. trichodermoides* M. Cabello & al. from a nest of *Mycocepurus goeldii* in São Paulo, Brazil (Masiulionis & al. 2015, Meirelles & al. 2015).

Additionally, Gerardo & al. (2006) isolated and characterized several *Escovopsis* strains from *Apterostigma* ants in Panama, Costa Rica, and Argentina, grouping the strains by coloration of mass conidia (brown, yellow, white, and pink), contrasting with the exclusively brown conidia of all described species of *Escovopsis*. No further details on the taxonomy of these morphotypes were mentioned, making it impossible to assign them formally to any *Escovopsis* species; but informally, and based on phylogenetic information, these morphotypes were considered members of *Escovopsis* (Gerardo & al. 2006). Brown conidial morphotypes were associated with the ant genera *Trachymyrmex*, *Apterostigma, Atta,* and *Acromyrmex;* white conidial morphotypes with *Apterostigma* and *Cyphomyrmex;* yellow conidial morphotypes with *Apterostigma;* and pink conidial isolates with *Cyphomyrmex* and *Apterostigma* (Gerardo & al. 2006).

The present work aims to describe, both morphologically and molecularly, new species of *Escovopsis* found in nests of *Acromyrmex* and *Atta* during a biodiversity study of the genus *Escovopsis* from different phytogeographical regions of Argentina.

Materials & methods

Fungal Isolates—Between June 2006 and November 2012, we sampled nests of leaf-cutting ants from different Argentinean provinces to isolate *Escovopsis* strains.

Twenty-six isolates of *Escovopsis* were obtained from garden material from different sites and different ant nests. Fungal isolations were made in the field and were conducted by collecting small individual pieces of fungus garden material (~2 mm^3) and placing them on PDA (Potato dextrose agar) plates with Penicillin–G (100 U/ml) with the pH of culture media adjusted to pH 7. Twenty samples from each nest were taken in situ from different areas of the cultivar garden. All isolates were taken to the laboratory and incubated 7–10 days at 25 ºC. After incubation, morphological distinct isolates were recovered and subcultured in additional plates in order to obtain pure cultures. *Escovopsis* isolates were maintained as live cultures in PDA until conidia and mycelia were observed and then preserved in glycerol (20% v/v) at –80 ºC. Holotypes were deposited as dried collections in the Herbarium, Universidad de Buenos Aires, Argentina (BAFC). Ex-holotype and additional strains examined were deposited in the culture collection of the Quilmes National University, Bernal, Buenos Aires, Argentina (UNQ).

Morphological analysis—All isolates were identified morphologically using macro- and microscopic characters. Isolates were grown on PDA and on modified Czapek yeast agar (mCYA) composed of 0.03 M KNO_3, 5.74×10^{-3} M K_2HPO_4, 6.7×10^{-3} M KCl, 2.02×10^{-3} M $MgSO_4 + 7H_2O$, 3.7×10^{-4} M $FeSO_4 + 7H_2O$, 8.7×10^{-6} M $ZnSO_4+7H_20$, 2×10^{-5} M $CuSO_4+5H_2O$, 5g/L Yeast Extract (DIFCO), 30g/L dextrose, 20g/L agar. After the cultures were incubated at 25 ºC, 80% RH, in darkness for 7 d, the colony diameter was measured using the software ImageJ V1.47. The coloration of the colony was coded according to Maerz & Paul (1930). For microscopic studies, fungal material was mounted in water and observed under a Nikon Eclipse E200 compound microscope; to improve visualization, staining with methylene blue, Congo red, and phloxine was used. The following microscopic characters were observed: shape, size, and ornamentation of conidia, shape and size of conidiogenous cell and vesicles, and diameter of vegetative hyphae. Each character was measured at least twenty times per isolate. To improve measurement precision, conidial size was measured using the software Micrometrics TM SE Premium 2.8234.

All isolate measurements were compared to those of the seven described species of *Escovopsis* in the literature. Strains that did not agree with any described species were clustered into morphotypes, which were also described. For *E. weberi* and *E. aspergilloides* we followed the original descriptions of Muchovej & Della Lucia (1990) and Seifert & al. (1995), as well as the commentaries of Augustin & al. (2013). Although previous *Escovopsis* studies refer to 'spores' (Gerardo & al. 2006, Masiulionis & al. 2015, Meirelles & al. 2015), we use the word 'conidia' as the correct terminology for the asexual reproductive structures.

DNA extraction—Total genomic DNA was extracted by a modification of the method published by Augustin & al. (2013). Isolates were cultured in PDA for seven days at 25 ºC and 80 % RH in darkness. Mycelia (50 mg) were placed in a mortar at −80 ºC with 500 µL of CTAB buffer (2% cetyltrimethyl ammonium bromide, 1.4 M NaCl at pH 8.0, 20 mM EDTA at pH 8.0, 2% Tris–Cl at pH 8.0 and 1% β–

mercaptoethanol). Using a ceramic pestle, fungal material was ground and later placed in a 1.5 ml microtube. Material was centrifuged for 30 s at 14,500 rcf and then 500 µL of chloroform were added, mixed and centrifuged 15 min at 14,500 rcf. Finally, the aqueous phase was transferred into a new microtube and one volume of cold isopropanol (−20 ºC) was added. Samples were precipitated overnight at −20 ºC and then centrifuged 15 min at 14,500 rcf to recover DNA. DNA pellets were air dried and resuspended in 50 µL of TE buffer (10 mM Tris–HCl pH 7.5; 0.1 mM EDTA). DNA concentration was determined by Nanodrop 2000 (Thermo Scientific), and integrity was examined in 0.8% agarose gels.

PCR amplification and sequencing—Two gene regions were analyzed: the nuclear D1/D2 domains of 28S rDNA gene and the TEF1 gene encoding translation elongation factor 1α (EF–1α). The 28S rDNA PCR was conducted with the primers CLA–F (5′–gcatatcaataagcggagga–3′) and CLA–R (5′–gactccttggtccgtgtttca–3′) (Currie & al. 2003); and the EF–1α using the primers EF1–5rl (5′–gtgataccacgctcacgctc–3′) and EF1–3F (5′–cacgtcgactccggcaagtc–3′) (Gerardo & al. 2004). Amplification of the 28S rDNA fragment was carried out in 50 µL with 1× PBL Taq Buffer, 3 mM $MgCl_2$; 0.2 mM of each dNTP; 1 U of Pegasus Taq; 0.4 µM of each primer and 75–100 ng DNA and purified H_2O to complete volume. PCR conditions were: 2 min at 95 ºC, 40 cycles with 30 s at 95 ºC, 60 s at 52 ºC, and 90 s at 72 ºC; and a final step with 5 min at 72 ºC. The amplification of TEF1 amplification was carried out in 50 µL with 1× PBL Taq Buffer, 3 mM of $MgCl_2$; 0.2mM from each dNTP; 1 U of Taq (Pegasus model); 0.4 µM of each primer (PBL company); 25–100 ng of DNA, and purified H_2O to complete volume. PCR conditions were: 2 min at 95 ºC, 40 cycles with 30 s at 95 ºC, 60 s at 52 ºC and 90 s at 72 ºC; and finally, 5 min at 72 ºC. All PCR reactions were done in a Veriti 96-well thermal cycler. PCR products were purified and sequenced by Macrogen Corporation.

Phylogenetic analyses—All sequences generated in this study and sequences available in GenBank were aligned using ClustalW (Thompson & al. 1994) and manually edited using MEGA 5 (Tamura & al. 2013). Additionally, poorly aligned portions and divergent positions (ambiguous positions) were deleted using CSIC-UPF Gblocks with less stringent options. Phylogenies were reconstructed using three approaches: maximum likelihood (ML), maximum parsimony (MP), and Bayesian inference (BI). For the analyses, complete deletion of gaps for 28S rDNA and partial deletion (80%) for TEF1 sequences were used.

For ML analysis, the DNA sequence evolution model was established based on the Akaike information criterion (AIC) implemented in MEGA 5. The Tamura–Nei model was selected for 28S rDNA with a Γ distribution to model evolutionary rate differences among sites (2 categories (+G, parameter = 0.3419)). For TEF1, the Kimura 2–parameter model with a Γ distribution to model evolutionary rate differences among sites (2 categories (+G, parameter = 1.4362)) was used. The initial tree for the heuristic search was obtained by applying the neighbour–joining method to a matrix of pairwise

distances estimated by the maximum composite likelihood (MCL) approach. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. Heuristic ML bootstrap analysis consisted of 1000 pseudoreplicates.

We ran MP analysis in MEGA 5 using heuristic searches with 300 random-addition sequence replicates. Tree-bisection reconnection (TBR) branch swapping was also performed. Heuristic MP bootstrap analysis consisted of 1000 pseudoreplicates (TBR branch swapping).

The BI analysis was performed in MrBayes v. 3.1 (Ronquist & Huelsenbeck 2003). We calculated posterior probabilities by a Metropolis–coupled Markov chain Monte Carlo analysis until the runs converged with a split frequency of 0.01. We employed the General Time Reversible model with a proportion of sites invariant and gamma–distributed rates (GTR+I+G). We used a sample frequency of 1000 independent analyses, with ten million generations each. The first 25% generations were discarded as "burn–in". The strict consensus trees and posterior probabilities were calculated from 15,000 trees.

The Figtree V1.4.2 program was used to obtain the final tree and to analyse the Bayesian probability.

Phylogenetic results

Among the 26 isolates studied, five matched the description of Muchovej & Della Lucia (1990) for *E. weberi.* In addition, five morphotypes presented marked differences from all previously described species. Morphological and phylogenetic analyses support these morphotypes as independent species. Among the most important characteristics, we can highlight the morphology and size of vesicles, conidial shape and size, and colony coloration in mCYA. Five new *Escovopsis* species are proposed: *E. atlas, E. catenulata, E. longivesica, E. primorosea,* and*, E. pseudoweberi.*

The matrix for the 28S rDNA gene comprised 38 sequences and 484 characters including 87 conserved sites, 397 variable sites, and 168 parsimony informative characters. On the other hand, the data matrix for the TEF1 gene comprised 53 sequences and 134 characters including 3 conserved sites, 134 variable sites, and 127 parsimony informative characters. All sequences were deposited in GenBank under accession numbers KU298275–KU298308. Alignments were deposited in TreeBASE under accession number 18631.

We obtained robust phylogenies for both markers, 28S rDNA and TEF1, showing no significant differences in the position of the main clades including *Escovopsis* reference sequences from GenBank and the sequences obtained in this study. Maximum likelihood trees are presented in Fig. 1 for 28S rDNA and in Fig. 2 for TEF1. Multilocus phylogenies were not possible using both markers because several isolates with TEF1 sequences (yellow, white, and pink

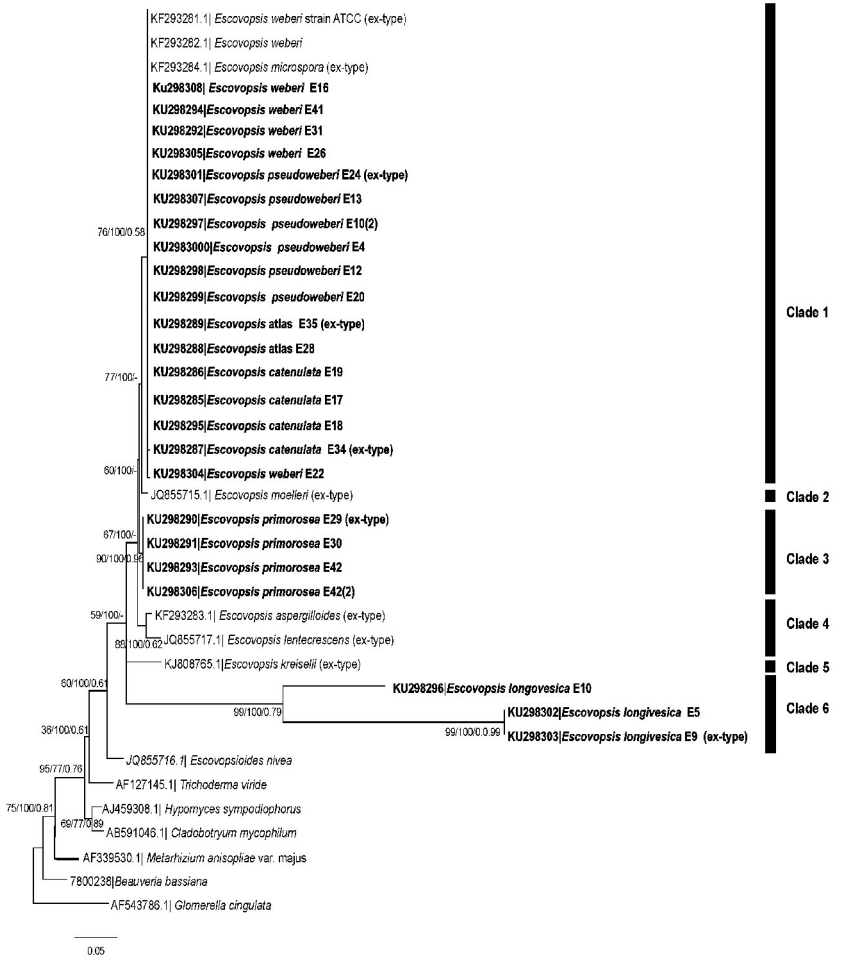

Fig. 1: Phylogeny of *Escovopsis* illustrating species relationships inferred from joint ML analysis of 28S rDNA sequence analysis. The series of three values at nodes correspond to ML and MP bootstrap values > 75%, and, BI posterior probabilities > 0.90.

conidiated clades and *E. trichodermoides*) were not available in GenBank for 28S rDNA.

Phylogenetic analyses of 28S rDNA gene (Fig. 1) support *Escovopsis* as a monophyletic group, where six clades were defined. Clade 1 included the type species of the genus, *E. weberi*, and closely related isolates, with varying

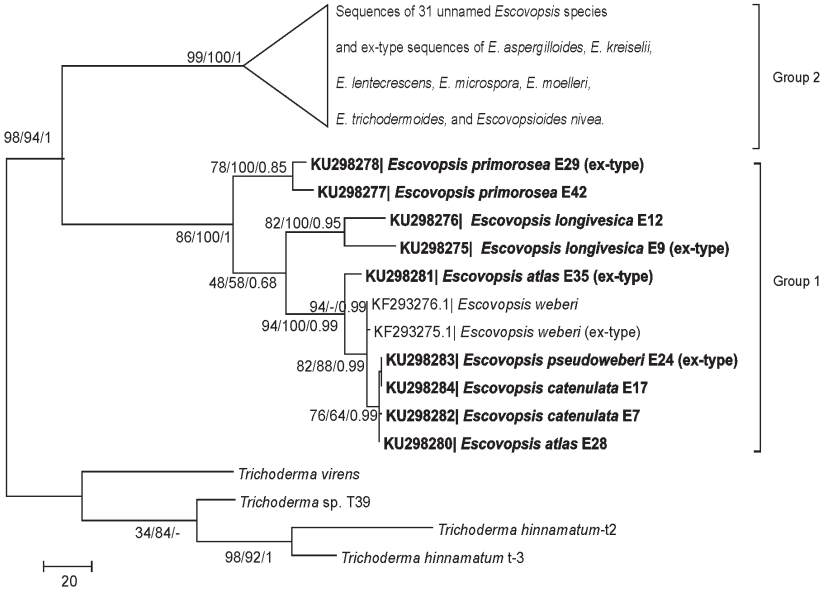

Fig. 2: Phylogeny of *Escovopsis* illustrating species relationships inferred from joint ML analysis of TEF1gene analysis. The series of three values above internal branches correspond to ML and MP bootstrap values > 75%, and BI posterior probabilities > 0.90.

levels of support (ML = 100%, MP = 76%, BI = 0.58). Clade 1 is composed of *E. weberi* (KF293281, KF293282), *E. microspora* (KF293284), *E. weberi* isolates from Argentina (E16, E41, E31, E22, E26), and representatives of three new species: *E. pseudoweberi* (E4, E13, E10(2), E12, E20), *E. atlas* (E28, E35), and *E. catenulata* (E17, E18, E19, E34). The 28S rDNA marker failed to separate these species into monophyletic groups presenting a single polytomy. Clade 2 is composed of a single reference sequence for *E. moelleri* (JQ855715) with high moderate support (ML = 77%, MP = 100%). Clade 3 grouped isolates E29, E30, and E42 of the new species *E. primorosea* in a well-supported monophyletic group (ML = 90%, MP = 100%, BI = 0.96). Clade 4 is formed by the reference sequences of *E. aspergilloides* (KF293283) and *E. lentecrescens* (JQ855717) with good support (ML = 88%, MP = 100%) but low posterior probability (BI = 0.62). Clade 5 is represented by a single lineage containing *E. kreiselii* (K808765); its relationship to other *Escovopsis* species is unresolved. Finally,

the basal Clade 6 appears as a well-supported group composed of isolates E5, E9, and E10 of the new species *E. longivesica* (ML = 99%, MP = 100%, BI = 0.79). In this phylogeny, the reference sequence of *Escovopsioides* (JQ8557161) did not group with any *Escovopsis* sequences.

In the phylogenetic analyses of the TEF1 gene (Fig. 2), the highly supported clade (ML = 86%, MP = 100%, BI = 1) of brown conidiated species, Group 1, contains *E. weberi* and all of our new *Escovopsis* species and several unidentified isolates from previous studies. Within Group 1, there were well-supported clades for *E. longivesica* (ML = 82%, MP = 100%, BI = 0.95) and *E. primorosea* (ML = 78%, MP = 100%, BI = 0.85), separating them from the other three new *Escovopsis* species from Argentina and from *E. weberi* (Fig. 2). *Escovopsis weberi* (the generic type), *E. atlas*, *E. pseudoweberi*, and *E. catenulata* were associated in a monophyletic group (ML = 94%, MP = 100%, BI = 0.99) with undetermined relationships. Group 2 is formed by a well-supported clade (ML = 99%, MP = 100%, BI = 1) containing the reference sequence (ex-type) of *E. aspergilloides* (AY172632), *E. kreiselii* (KJ808766), *E. lentecrescens* (JQ855714), *E. microspora* (KJ935030), *E. moelleri* (JQ855712), *E. trichodermoides* (KF033128), *Escovopsioides nivea* (JQ855713) and several sequences from unidentified *Escovopsis*.

Taxonomy

Escovopsis primorosea Marfetán, A.I. Romero & Cafaro, **sp. nov.** Fig 3, 8 d

MycoBank MB 816915

Differs from other *Escovopsis* species by its pink colony coloration on mCYA medium, and its simultaneous production of cylindrical, claviform (solitary and catenulate), and subglobose vesicles.

Type—Argentina, Tucumán, Parque Nacional Campo de los Alisos, (27°13'51"S 65°54'46"W), 1500 m asl, fungal garden of *Acromyrmex aspersus* F. Smith, Nov 2012, J.A Marfetán E29 (**Holotype,** BAFC-H 52761; culture ex type culture, UNQ E29; GenBank KU298290, KU298278).

Etymology—In reference to the coloration of the colony and conidia on mCYA, which is pink initially, but later becomes brown.

Colonies fast growing, colony diameter 5.28–7.04 cm after 7 days on PDA. On mCYA, colony white turning pale pink after four days, then light brown at five days. In PDA, colony white becoming light brown after seven days. Hyphae hyaline, smooth, septate, with Woronin bodies highly visible, 6.2–24.7 µm diam. Conidiophores hyaline, orthogonal branched, cylindrical. Vesicles cylindrical to claviform, with a rounded apex, 15–62.5 (–81.25) × 6.25–26.25 µm and occasionally catenulate, generally with two vesicles

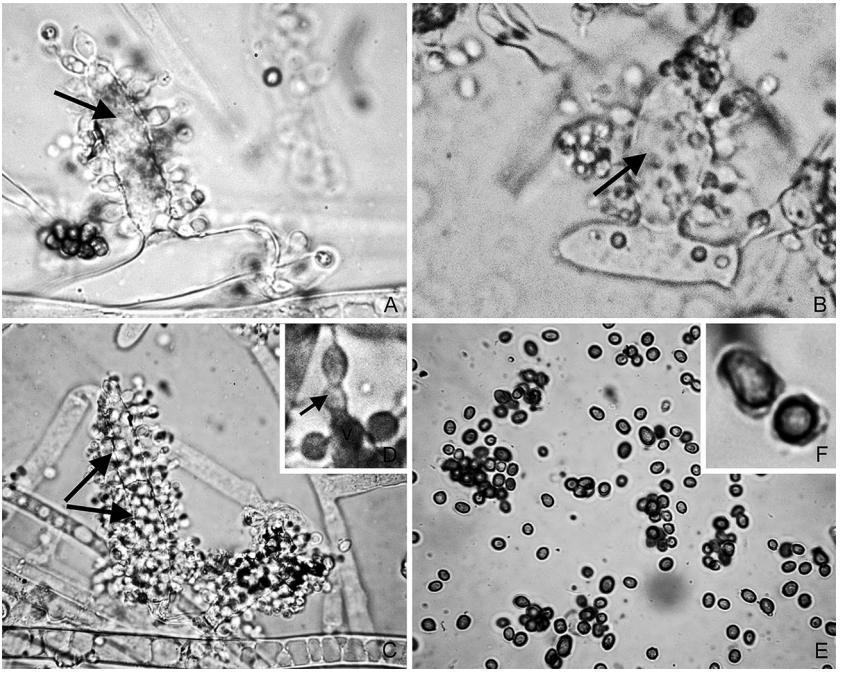

Fig. 3: *Escovopsis primorosea* (holotype, BAFC-H 52761). A. Conidiophore (arrow); B. Vesicle (arrow); C. Catenulate vesicles (arrow); D. Support cell separate (arrow) from the vesicle by septum (v = vesicle); E. Conidia; F. Conidia with cap-like structure.

(12.5–62.5 × 6.25–21.25 µm), occasionally ovoid to subglobose, simple vesicles, 15–27.5 × 12.5–26.25 µm and rarely catenulate, generally with two vesicles. Conidiogenous cells phialidic, with rounded base, elongated neck and inconspicuous collarette, discrete, hyaline, 2.5–12.5 × 1.25–3.74 µm, at the apex of the vesicle conidiogenous cells over a hyaline and octagonal supporting cell (3.1–5 × 5–6.25 µm). Conidia with basipetal development, catenulate, ovoid to ellipsoid, 2.5–5 × 2.5–6.25 µm, hyaline becoming brown later, cap–like structure present.

Additional specimens examined—**ARGENTINA**, **Tucumán**, Parque Nacional Campo de los Alisos, 27°13′51″S 65°54′47″W, 1500 m asl, fungal garden of *Acromyrmex aspersus*, Nov 2012, J.A. Marfetán E30 (UNQ E30; GenBank KU298291, KU298279); J.A. Marfetán E42 (UNQ E42; GenBank KU298293, 298277); J.A. Marfetán E42(2) (UNQ E42(2); GenBank KU298306).

Commentary—*Escovopsis primorosea* can be easily distinguished from the other species by its pink coloration in mCYA and by the simultaneous presence

of cylindrical, claviform (solitary and in chains), and subglobose vesicles. This species can also be diagnosed by 28S rDNA and TEF1 genes.

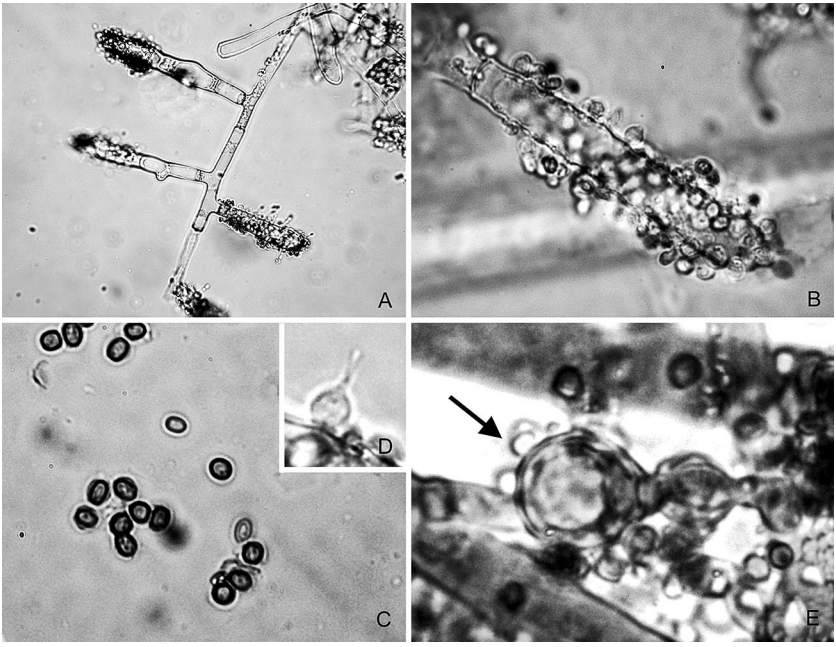

Fig. 4: *Escovopsis longivesica* (holotype, BAFC-H 52762). A, B. Vesicles; C. Conidia; D. Conidiogenous cell; E. Chlamydospore.

Escovopsis longivesica Marfetán, A.I. Romero & Cafaro, **sp. nov.** Figs 4, 8a

MycoBank MB816916

Differs from other *Escovopsis* species by its longer vesicles.

Type—Argentina, La Pampa, Parque Nacional Lihuel Calel, 38°00′06″S 65°35′42″W, 325 m asl, fungal garden of *Acromyrmex striatus* Roger, Oct 2012, J.A. Marfetán E9 (**Holotype**, BAFC-H 52762; ex-type culture, UNQ E9; GenBank KU298303, KU298275).

Etymology—In reference to the conidiophores with longer vesicles.

Colonies in PDA, colony diameter 4.3–7.8 cm after 7 days, initially white in color becoming brown after seven days, abundant aerial mycelium. On mCYA with similar morphology as above. Hyphae hyaline, septate, 3.7–20 µm diam. Conidiophores hyaline growing from the aerial mycelium, with cylindrical, orthogonal branched. Vesicles mainly cylindrical, occasionally

claviform, 6.2–20 × 18.7–62.5 (–75) µm, with a rounded apex, solitary and terminal. Conidiogenous cells phialidic, with rounded base and elongated neck and inconspicuous collarette, 2.5–5 × 2.5–15 µm. Conidia catenulate, with basipetal development, subglobose to ovoid (occasionally ellipsoid), 1.17–2.87 × 1.7–5 µm, hyaline becoming brown later, thick–walled, cap–like structure present. Chlamydospores occasionally present, globose to subglobose, 11.5–12.2 × 11.1–12.7 µm.

Additional specimens examined— **ARGENTINA**, **Buenos Aires**, Mercedes, 34°39′44″S 59°27′07″W, 38 m asl, fungal garden of *Acromyrmex lundii* Guérin-Méneville, Sep 2008, J.A. Marfetán E10 (UNQ E10; GenBank KU298296); Gonnet, 34°52′46″S 58°00′41″W, 3 m asl, fungal garden of *Acromyrmex lundii*, May 2009, J.A. Marfetán E5 (UNQ E5; GenBank KU298302). **La Pampa**, Toay, 36°32′42″S 64°03′01″W, 174 m asl, fungal garden of *Acromyrmex striatus*, Oct 2011, J.A. Marfetán E12 (UNQ E12; GenBank KU298276).

Commentary—*Escovopsis longivesica* is morphologically easily distinguished from other described species by the greater length of its vesicles. This species is separated from *E. aspergilloides, E. lentecrescens,* and *E. primorosea* by the absence of globose to subglobose vesicles. In addition, it is distinguished from *E. weberi, E. moelleri,* and *E. microspora* by the vesicle length (longer in *E. longivesica*) and conidial size (bigger in *E. longivesica*). This species can also be distinguished from the other species using 28S rDNA and TEF1 sequences.

Escovopsis pseudoweberi Marfetán, A.I. Romero & Cafaro, **sp. nov.** Figs 5, 8f

MycoBank MB 816917

Differs from *Escovopsis weberi* and *E. microspora* by its smaller conidia.

Type— Argentina, Tucumán, Parque Nacional Campo de los Alisos, 27°13′50″S 65°55′51″W, 1500 m asl, fungal garden of *Acromyrmex aspersus*, Nov 2012, J.A. Marfetán E24 (**Holotype**, BAFC-H 52763; ex-type culture, UNQ E24; GenBank KU298301, KU298283).

Etymology—Fake *E. weberi,* due to close similarities with *E. weberi.*

Colonies in PDA, colony diameter 5.2–7.95 cm after 7 days, initially white in color becoming brown after five days. Hyphae hyaline, smooth, septate, 3.75–17.5 µm diam, Woronin bodies conspicuous. Conidiophores terminal, hyaline, with cylindrical cells and orthogonal ramifications, bearing numerous vesicles covered by conidiogenous cells. Vesicles claviform, occasionally cylindrical, with a rounded apex 6.2–21.2 × 20–66.2 µm, developing laterally and in the apex of the conidiophores. Conidiogenous cells with rounded base and elongated neck and inconspicuous collarette, phialidic (2.5–5 ×

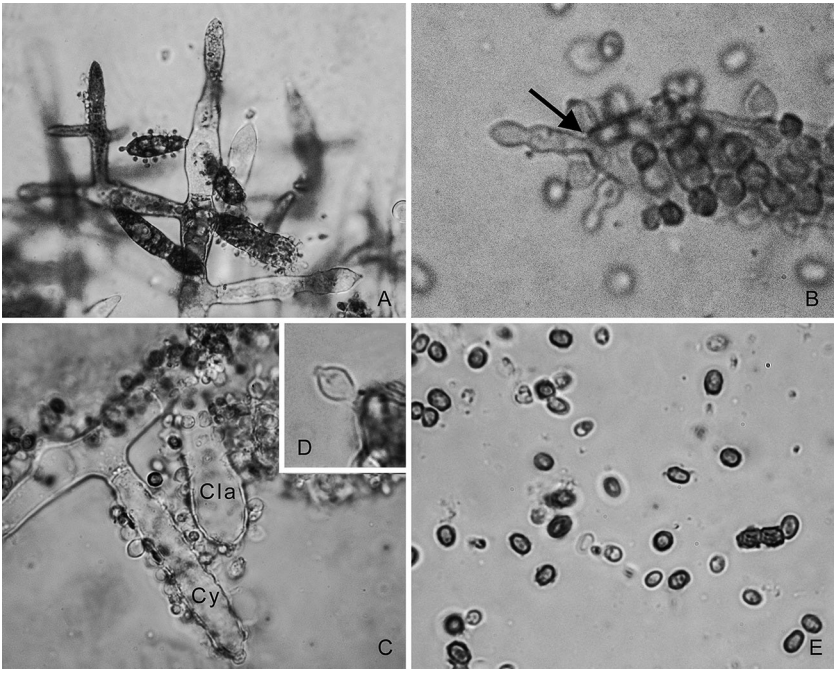

Fig. 5: *Escovopsis pseudoweberi* (holotype, BAFC-H 52763). A. Conidiophores; B Conidiogenous cell with a supporting cell (arrow); C. Cylindrical (Cy) and claviform (Cla) vesicles; D. Conidiogenous cell; E- Conidia.

3.7–12.5 µm), occasionally in the vesicle apex subulate or lageniform (14.5 × 3.75–5 µm) over a refinement at the apex of the vesicle. Conidia catenulate, with basipetal development, subglobose to ovoid, sometimes ellipsoid, 0.9–2.7 × 1.2–4.2 µm, hyaline becoming brown with days, rough–walled, cap–like structure occasionally present.

Additional specimens examined—**ARGENTINA**, **Corrientes**, Mercedes, 29°11′50″S 58°02′28″W, 98 m asl, fungal garden of *Acromyrmex lundii*, Mar 2008, J.A. Marfetán E10(2) (UNQ E10(2); GenBank KU298297); J.A. Marfetán E13 (UNQ E13; GenBank KU298307); Mercedes, 29°11′57″S 58°02′18″W, 98 m asl, fungal garden of *Acromyrmex heyeri* Forel, Mar 2008, J.A. Marfetán E12 (UNQ E12; GenBank KU298298). **Santa Fe**, San Cristobal, 30°22′56″S 61°14′16″W, 67 m asl, fungal garden of *Acromyrmex lobicornis* Emery, Aug 2008, J.A. Marfetán E20 (UNQ E20; GenBank KU298299). **Buenos Aires**, Gonnet, Estación de Cría de Animales Silvestres (ECAS), 34°50′44′S 58°07′05″W, 3 m asl, fungal garden of *Acromyrmex lundii*, Mar 2009, J.A. Marfetán E4 (UNQ E4; GenBank KU298300).

Commentary—*Escovopsis pseudoweberi* is very similar to *E. weberi* and to *E. microspora* but is distinguished from them by its smaller conidia. Conidial size ranges are 2.5–3.5 × 3.5–4.5 µm for *E. weberi* and (1.5–)2–3 × (2–)2.5–3 µm for *microspora* with *E. pseudoweberi* presenting the smallest range. *Escovopsis pseudoweberi* can also be distinguished from other species using 28S rDNA and TEF1 sequences.

Escovopsis atlas Marfetán, A.I. Romero & Cafaro, **sp. nov.** Figs 6, 8c

MycoBank MB 816918

Differs from other *Escovopsis* species by its subglobose vesicles supported by an octagonal cell.

Type—Argentina, Tucumán, Parque Nacional Campo de los Alisos, 27°13′51″S 65°54′47″W, 1500 m asl, fungal garden of *Acromyrmex aspersus*, Nov 2012, J.A. Marfetán E35 (**Holotype**, BAFC-H 52764; ex-type culture, UNQ E35; GenBank KU298289, KU298281).

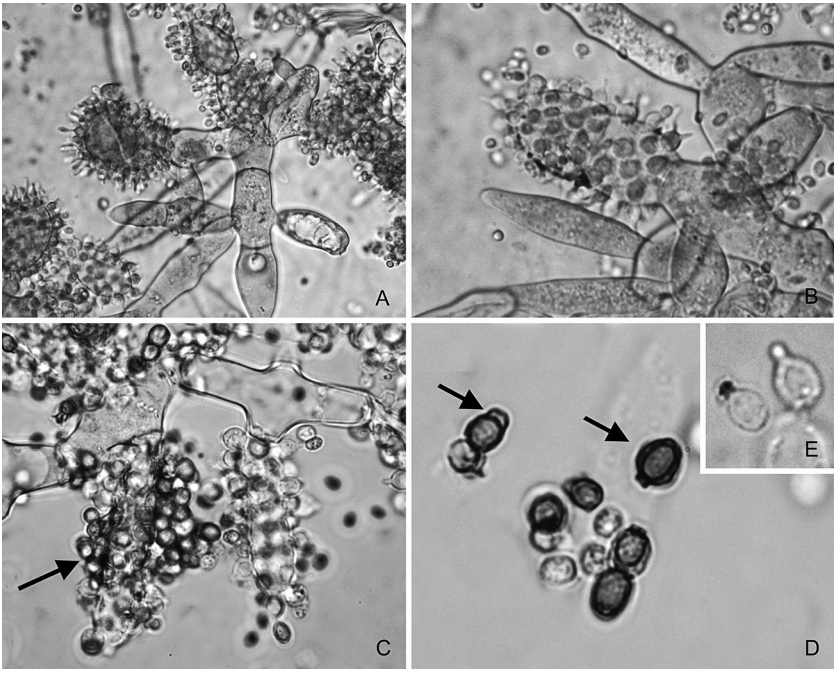

Fig. 6: *Escovopsis atlas* (holotype, BAFC-H 52764). A. Conidiophore (arrow); B. Catenulate subglobose vesicles separated (arrow); C. Cylindrical vesicles; D. Conidia with cap-like structure (arrow); E. Conidiogenous cells.

Etymology—Greek, *Atlas*, a divinity who bears the globe on his shoulders. This is a reference to the subglobose vesicles (= globe) supported by an octagonal cell (= shoulders).

Colonies in PDA, colony diameter 5.5–7.9 cm at day 7, initially white in color becoming brown after 5 days, abundant aerial mycelium. Hyphae hyaline, septate, 6.25–5 µm diam, with Woronin bodies highly visible. Conidiophores, hyaline growing from the aerial mycelium, cylindrical cells and orthogonal branched. Vesicles subglobose (8.8–27.5 × 16.2–31.2 µm) and claviform (18.7–52.5 × 7.5–30 µm), occasionally subglobose catenulate (18.7–23.7 × 20–21.7 µm), supported by octagonal cells (19.3–30 × 23.3–40.5 µm). Conidiogenous cells phialidic, with rounded base, elongated neck and inconspicuous collarette, 2.5–5 × 3.7–8.7 µm. Conidia catenulate, with basipetal development, cylindrical to ellipsoidal, 1.9–3.7 × 2.5–6.3 µm, hyaline becoming brown with days, thick walled.

Additional specimen examined—**ARGENTINA**, **Salta**, Parque Nacional El Rey, 25°13′59″S 64°42′58″W, 900 m asl, fungal garden of *Acromyrmex lundii*, Nov 2012, J.A. Marfetán E28 (UNQ E28; GenBank KU298288, KU298280).

Commentary—The subglobose vesicles of *E. atlas* easily separate it from *E. weberi, E. moelleri, E. microspora,* and *E. longivesica*. *Escovopsis atlas* is distinguished from *E. lentecrescens* by growth rate in PDA, from *E. aspergilloides* by smaller subglobose vesicles and conidia, and from *E. primorosea* by conidial coloration (pink) in mCYA.

Escovopsis catenulata Marfetán, A.I. Romero & Cafaro, **sp. nov.** Figs 7, 8e

MycoBank MB 816919

Differs from other *Escovopsis* species by its catenulate claviform vesicles.

Type—Argentina, Tucumán, Parque Nacional Campo de los Alisos, 27°13′48″S 65°54′51″W, 1500 m asl, fungal garden of *Acromyrmex aspersus*, Nov 2012, J.A. Marfetán E34 (**Holotype**, BAFC-H 52765; ex-type culture UNQ E34; GenBank KU298287).

Etymology—from the Latin *catenulatus* "forming short chains" referring to the production of vesicles in chains.

Colonies in PDA, colony diameter 6.2–9.2 cm after 7 days, initially white in color becoming light brown in the older part of the colony, abundant aerial mycelium. Hyphae hyaline, cylindrical, smooth, 3.7–13.7 µm diameter, with Woronin bodies highly visible. Conidiophores terminal, hyaline, growing from the aerial mycelium, cylindrical, orthogonal branched. Vesicles solitary or catenulate (2-4), cylindrical, occasionally claviform, 15–75 × 6.2–22.5 µm. Conidiogenous cells phialidic, with rounded base, elongated neck and

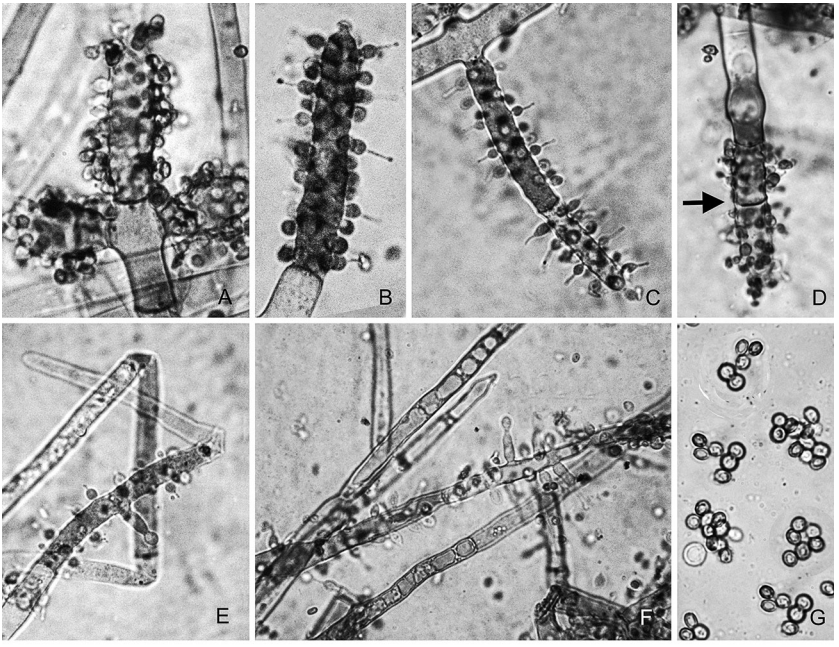

Fig. 7: *Escovopsis catenulata* (holotype, BAFC-H 52765). A. Conidiophore; B. Vesicles; C, D. Catenulate vesicles; E, F. Intercalary vesicle; G- Conidia.

inconspicuous collarette, 2.5–6.2 × 12.5–12.5 µm. Conidia catenulate, with basipetal development, subglobose to ovoid 1.22–6.25 × 1.6–6.25 µm, hyaline becoming brown later. Chlamydospores occasionally present in terminal chains, 7.95–17.4 × 12.3–17.3 µm.

Additional specimens examined—**ARGENTINA**, **Corrientes**, Mercedes, 29°11′53″S 58°02′28″W, 98 m asl, fungal garden of *Acromyrmex lobicornis*, Mar 2008, J.A. Marfetán E17 (UNQ E17; GenBank KU298285, KU298284). **Santa Fe**, San Cristobal, 30°22′55″S 61°14′20″W, 67 m asl, fungal garden of *Atta vollenweideri* Forel, Aug 2008, J.A. Marfetán E18 (UNQ E18; GenBank KU298295); San Cristobal, 30°22′57″S 61°14′16″W, 67 m asl, fungal garden of *A. heyeri*, Aug 2008, J.A. Marfetán E19 (UNQ E19; GenBank KU298286). **Buenos Aires**, La Plata, 34°54′39″S 57°56′05″W, 10 m asl, fungal garden of *Acromyrmex lundii*, Apr 2009, J.A. Marfetán E7 (UNQ E7; GenBank KU298282).

Commentary—*Escovopsis catenulata* is readily separated from *E. aspergilloides, E. lentecrescens,* and *E. atlas* by the absence of globose or subglobose vesicles. It is distinguished from *E. weberi, E. pseudoweberi, E. longivesica, E. microspora,* and *E. moelleri* by the presence of catenulate vesicles. Finally, *E. catenulata*

is separated from *E. primorosea,* which also presented catenulate cylindrical vesicles, by conidia coloration (pink in *E. primorosea*) in mCYA. Differentiation from *E. aspergilloides, E. kreiselii, E. lentecrescens, E. longivesica, E. microspora, E. moelleri,* and *E. primorosea* can be done using TEF1 and 28S rDNA sequences.

Escovopsis weberi J.J. Muchovej & Della Lucia, Mycotaxon 37: 192, 1990. Fig. 8b

Although our isolates identified as *E. weberi* agreed with the description by Muchovej & Della Lucia (1990), they showed some differences in size of particular structures. The vesicles in our isolates are wider (5–25 × (6.25–) 25–66.25 µm) than previously described (11–14 × 43–58 µm), and the conidia size range is also wider (2.5–5 × 2.5–6.25 µm) than the original description (2–3 × 2.2–3.3 µm). These data could represent normal morphological variation within the species; thus, we conservatively assigned our isolates to *E. weberi*.

Specimens examined—**ARGENTINA**, **Santa Fe**, San Cristóbal, 30°22′54″S 61°14′15″W, 67 m asl, (UNQ E16; GenBank KU298308). **La Pampa**, Santa Rosa 36°36′58″S 64°18′51″W, 177 m asl (UNQ E22; GenBank KU298304); Toay, 36°32′42″S 64°03′01″W, 174 m asl (UNQ E26; GenBank KU298305). **Salta**, El Rey, , 25°13′59″S 64°42′48″W, 900 m asl, (UNQ E31; GenBank KU298292); , 25°13′61″S 64°42′53″W, 900 m asl, (UNQ E41; GenBank KU298294).

Key to species of *Escovopsis*

1a. Conidiophores with vesicles *2*
1b. Conidiophores without vesicles 11

2a. Pink coloration in modified CYA media after 4 days ..... *E. primorosea*
2b. Brown coloration in modified CYA media after 4 days 3

3a. Subglobose or globose vesicles present 4
3b. Subglobose or globose vesicles absent 6

4a. Colony growth ≤0.7 cm diam after 14 days ..... *E. lentecrescens*
4b. Colony growth ≥0.7 5

5a. Subglobose vesicles (8.75–27.5 × 16.25–31.25 µm) ..... *E. atlas*
5b. Globose to subglobose vesicles (20 × 35 µm) ..... *E. aspergilloides*

6a. Catenulate vesicles present ..... *E. catenulata*
6b. Catenulate vesicles absent 7

7a. Conidia 3–3.5 × 7–10 µm ..... *E. moelleri*
7b. Conidia <7 µm long 8

8a. Vesicle >70 µm long ..... *E. longivesica*
8b. Vesicle <70 µm long 9

9a. Vesicle up to 6–7 µm diam, conidia 1.5–3 × 2–3 µm ..... *E. microspora*
9b. Vesicle generally bigger 10

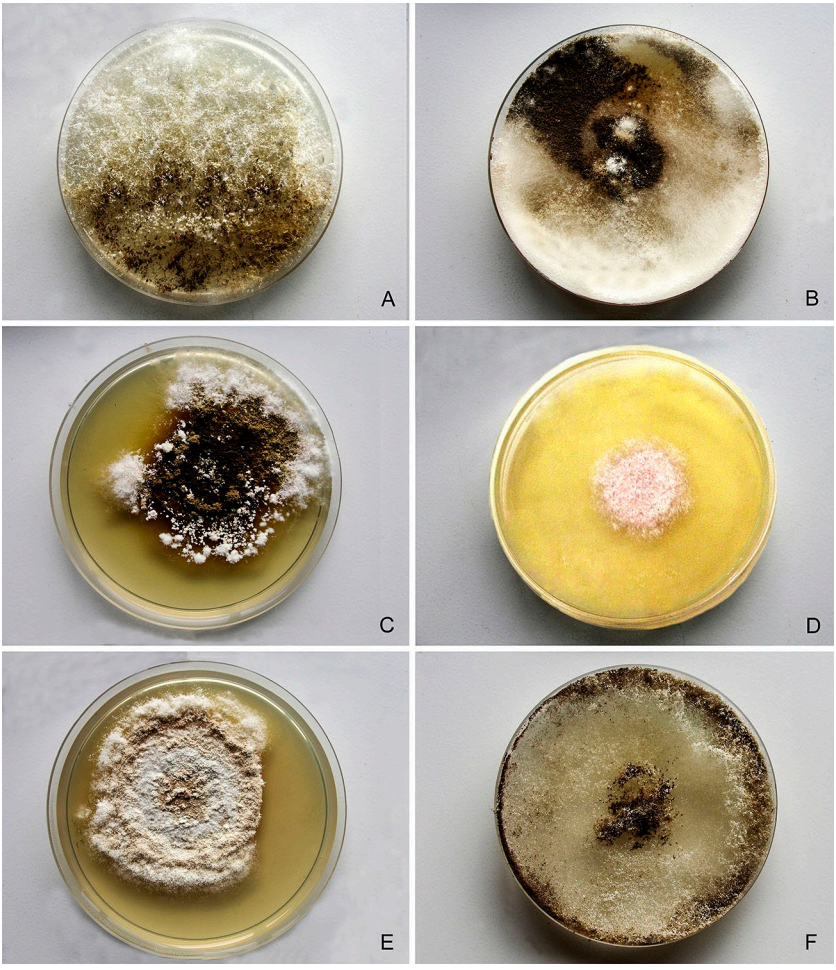

Fig. 8: Comparison of cultural characteristics of the six sequenced species in modified CYA media after 4 days: A. *Escovopsis longivesica*; B. *E. weberi;* C. *E. atlas*; D. *E. primorosea*; E. *E. catenulata*; F. *E. pseudoweberi.*

10a. Vesicles 5–20 µm diam, conidia 0.97–2.71 × 1.25–4.22 µm ..... *E. pseudoweberi*
10b. Vesicles 5–20 µm diam, conidia 2–5 × 2.2–6.25 µm ..... *E. weberi*
11a. Percurrent conidiogenous cells present ..... *E. kreiselii*
11b. Conidiophores *Trichoderma*-like; yellow soluble pigment present in PDA..... *E. trichodermoides*

Discussion

In Argentina, *Escovopsis* isolates have been reported from Santa Fé, Misiones, and Chaco Provinces (Folgarait & al. 2011; Taerum & al. 2007, 2010) and *E. weberi* isolates from Corrientes, Buenos Aires, and Santa Fé Provinces (Folgarait & al. 2011, Marfetán & al. 2015), but the scope of these studies focused on ecological and biological issues rather than taxonomy. Ours is the first study that describes *Escovopsis* isolates from Argentina at the species level using morphological and molecular data. Furthermore, it represents the most complete survey to date in Argentina (26 isolates) and one with the highest number of isolates spanning wide geographic (6 provinces) and host ranges (6 leaf-cutting ant species).

All the described species are well delimited by characters with clear diagnostic features that allow separation among species. Vesicle morphology and size and conidial shape and size are, as in other *Escovopsis* species, the most useful key characters. Additionally, we propose colony coloration in mCYA as a new diagnostic character within the genus.

Phylogenetic analyses using TEF1 and 28S rDNA presented robust results showing well supported clades for *Escovopsis longivesica* and *E. primorosea* isolates, supporting the hypothesis that these isolates belong to new species. The same analyses place *E. atlas, E. catenulata,* and *E. pseudoweberi* in the same clade with previously described species (Fig. 2) including *E. weberi* and *E. microspora* (Fig. 1). Although our results support the proposal of new *Escovopsis* species, it is clear that the molecular markers used are not enough to resolve species relationships. The lack of resolution using 28S rDNA and TEF1 genes is not new for *Escovopsis* species separation (Augustin & al. 2013). Previous work showed that these markers were unable to separate *E. weberi* and *E. microspora* (Augustin & al. 2013) even though in our 28S rDNA analysis we indeed were able to distinguish both species (Fig. 1). Limited taxon sampling may explain previous results for the non-Argentine species. Furthermore, this study confirmed that these markers are highly conserved and failed to resolve the phylogenetic relationships between some *Escovopsis* species, thus warranting evaluation of additional molecular markers.

Our data (Fig. 2) showed that the brown conidia clade was formed by *E. weberi* (ex-type), *E. aspergilloides, E. microspora, E. moelleri, E. lentecrescens*, and other unidentified associated isolates, while the white, pink, and yellow lineages were more closely associated with *Escovopsioides* than with *Escovopsis*. These results agreed with previous works (Masiulionis & al. 2015, Meirelles & al. 2015).

Surprisingly, our results showed that *E. trichodermoides* and *E. kreiselii* are more related to the white and pink conidiated clade than with the brown conidiated species (Fig. 2). In this sense, these two species have marked differences with the rest. Both species present holoblastic solitary conidia and conidiophores without vesicles (Masiulionis & al. 2015, Meirelles & al. 2015). These results could suggest that undescribed strains/isolates in these clades (pink, yellow, and white conidiated) could present additional differences to the known *Escovopsis* species. It is necessary to evaluate taxonomically the undescribed strains/isolates in these three clades to create a formal description of these interesting isolates and estimate their morphological variability in the genus. Moreover, in future studies scanning electron microscopy might be used to evaluate if there is any difference between the conidial ontogeny seen in *E. trichodermoides* and in the conidiogenous cells of the remaining species.

The present data showed no species-specific association between ant species and *Escovopsis* species, agreeing with previous works including bioassays showing that *Escovopsis* species can parasitize more than one leaf-cutting ant species (Folgarait & al. 2011, Taerum & al. 2007). In our study, *E. weberi* and *E. longivesica* were both isolated from *A. lundii* and *A. striatus* nests, indicating that these two species can infect different ant species. The same seems true for *E. atlas* isolated from *A. lundii* and *A. aspersus,* while *E. catenulata* and *E. pseudoweberi* were isolated from several different ant species. Furthermore, *E. catenulata* was isolated from ants in different genera, *Atta vollenweideri* and four *Acromyrmex* ant species contradicting any kind of specificity.

Our phylogenetic analyses (Fig. 2) did not show a particular phylogeography pattern among *Escovopsis* species and isolates. Although species from Argentina appear to be more closely related to species from Brazil than from other parts of South America, they are more closely associated with ant groups than with geographical site. However, our results suggest that temperate areas (southern Argentina) have a more restricted *Escovopsis* diversity (2 species) than in tropical zones (northern Argentina, Brazil, and Panama, with 11 species and several undescribed isolates). Species distribution studies, including an exhaustive identification of *Escovopsis* isolates, are needed to better understand which factors influence diversity and distribution and to build stronger hypotheses about its biogeography.

Evidence for additional species can be found in this work and others (Augustin & al. 2013) in comparing *E. weberi* isolates to the original description by Muchovej & Della Lucia (1990). There are still unidentified Argentine *Escovopsis* strains (data not shown) that are hardly distinguishable

from *E. weberi*. Finally, *Escovopsis* species are well delimited by morphological character correlation. However, for some taxa, the molecular markers used in this study have proved inadequate in satisfactorily solving the phylogenetic relationships at the species level. Further research on this matter is needed to develop a robust phylogenetic framework for *Escovopsis*.

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